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Storage and oxidation of long-chain fatty acids in the C57/BL6 mouse heart as measured by NMR spectroscopy

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Abstract **Triglyceride turnover in the isolated C57/BL6 mouse heart was measured by dynamic ^{13}C edit-^{1}H observe NMR and the rate of fatty acid oxidation was determined by ^{13}C NMR isotopomer analysis. In the presence of a physiological mixture of substrates, energy was produced in the citric acid cycle by oxidation of long-chain fatty acids (18%), ketones (34%), lactate (24%), pyruvate (7%), and other sources (17%). Exogenous fatty acids appeared in the triglyceride pool at 0.24 μmol/g dry wt/min, similar to the rate of oxidation of long-chain fatty acids, 0.16 μmol/g dry wt/min. Isoproterenol decreased the rate of de novo triglyceride synthesis and increased the rate of fatty acid oxidation.**

Keywords: Carbon-13; Heart metabolism; Fatty acids; Triglycerides; Isotopomer analysis; Citric acid cycle

1. Introduction

The main pathways of long-chain fatty acid metabolism in the heart are well-defined. After transport across the sarcolemma, long-chain fatty acids are activated by fatty acyl-CoA synthase and the resulting long-chain fatty acyl-CoA is either converted to fatty acyl carnitine by carnitine palmitoyl transferase I and subsequently oxidized in the mitochondria or esterified to triglyceride by glycerolphosphate acyl-transferase [1]. It has long been known that this triglyceride pool can be mobilized quickly by the normal heart [2,3], and abnormal triglyceride content has been described in numerous diseases including ischemia [4,5], diabetes [6] and mouse models of obesity [7]. However, it is only recently that causal links between abnormal triglyceride content and left ventricular dysfunction [7], muscle insulin resistance [8], or apoptosis [9] have been postulated.

In spite of the potential importance of endogenous triglycerides in disease, the kinetics of triglyceride turnover and the partitioning of fatty acids between storage and oxidation have not been widely examined. One experimental approach, biochemical measurement of triglycerides in tissue extracts, is not suitable for monitoring triglyceride turnover or for studies in functioning hearts. Therefore, many investigations of cardiac triglyceride turnover relied on monitoring glycerol release [10,11] or by detection of $^{14}CO_2$ after prelabeling of the endogenous lipid pools with radioactive fatty acids [12,13]. More recently, dynamic ^{13}C NMR was used to directly detect de novo synthesis of cardiac triglycerides from enriched palmitate [14]. Nevertheless, the overall balance between lipolysis and synthesis in response to simple interventions such as catecholamines remains uncertain. For example, lipolysis as measured by glycerol release or reduction of triglyceride mass is stimulated by catecholamines [11,12], but isoproterenol also increases triglyceride synthesis and causes accumulation of myocardial triglycerides in vivo [15,16].

The ability to non-invasively monitor triglyceride synthesis and degradation in the heart and other tissues without radiotracers would have wide clinical and research applications. The goal of the present study was to determine the fate of long-chain fatty acids entering a C57/BL6 mouse heart supplied with a physiologically relevant mixture of substrates. Since NMR detection of triglycerides by ^{1}H spectroscopy by NMR is more sensitive than direct ^{13}C detection, an inverse detection method was applied. Conventional ^{13}C NMR isotopomer analysis was used to determine the rate of oxidation of fatty acids by hearts and the fraction of cardiac triglycerides enriched in ^{13}C was determined by the spin-coupled satellites observed in ^{1}H NMR spectra of extracted triglycerides. Fatty acids entering the triglyceride pool were followed directly with high temporal resolution to determine the rate of triglyceride turnover as well as dynamic response to the β agonist, isoproterenol.

2. Materials and methods

2.1. Materials

[1,3-$^{13}C_2$]Ethyl acetoacetate, [3-^{13}C]sodium lactate, [U-$^{13}C_3$]sodium lactate, [3-^{13}C]sodium pyruvate, and a mixture of [U-^{13}C]long-chain fatty acids were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Triglyceride assay kits and glycerol standard were obtained from Sigma Chemical Co. (St. Louis, MO). Sep-Pak Vac RC cartridges were obtained from Waters Co. (WAT036950, Milford, MA). DL-Isoproterenol hydrochloride and all other chemicals were

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obtained from Sigma. C57/BL6 mice were obtained from Charles River Laboratories (Wilmington, MA) and given free access to standard mouse chow and water.

2.2. Heart perfusions

Protocols were approved by the Institutional Animal Care and Use Committee. Hearts from adult female mice were rapidly excised under general anesthesia and perfused using standard Langendorff methods at 37 °C and 100 cm H_2O. The modified Krebs–Henseleit (KH) buffer was bubbled continuously with a 95/5 mixture of O_2/CO_2, and contained 118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 8.2 mM glucose, 0.13 mM glycerol, 4.9 mM lactate, 0.49 mM pyruvate, 0.28 mM acetoacetate, 0.63 mM long-chain fatty acids, and 2% albumin. These are the average plasma substrate concentrations reported for resting mice [17–25]. The heart rate was monitored through an open-ended cannula in the left ventricle.

2.3. Isotopomer analysis from tissue extracts

Eight hearts were supplied for 45 min with non-recirculating medium containing [U-^{13}C]long-chain fatty acids, [3-^{13}C]lactate, [3-^{13}C]pyruvate, and [1,3-$^{13}C_2$]acetoacetate. As a result, [1,2-$^{13}C_2$]acetyl-CoA will be generated only from the fatty acids. Lactate and pyruvate will both produce [2-^{13}C]acetyl-CoA, acetoacetate will provide [1-^{13}C]acetyl-CoA, and unenriched acetyl-CoA can arise from utilization of glucose, glycerol, and/or endogenous substrates. Three additional hearts were perfused using the same protocol and enrichment patterns with the addition of 1 μM isoproterenol to the buffer. In a third group, eight hearts were supplied with medium containing [U-^{13}C]long-chain fatty acids, [U-$^{13}C_3$]lactate, [3-^{13}C]pyruvate, and [1,3-$^{13}C_2$]acetoacetate. In this series the fatty acids and lactate will both generate [1,2-$^{13}C_2$]acetyl-CoA. Since [3-^{13}C]pyruvate is the only source of [2-^{13}C]acetyl-CoA, the oxidation of lactate and pyruvate can be determined separately. Oxygen consumption was determined using coronary flow measurements and the difference in pO_2 between the arterial perfusate and the coronary effluent (Instrumentation Laboratory, Lexington, MA). Hearts were freeze-clamped after 45 min and stored at −70 °C. Two hearts perfused using the same conditions were combined to increase the amount of glutamate. Tissue was extracted with perchloric acid and resuspended in 0.6 mL ^2H_2O for NMR spectroscopy [26]. Citric acid cycle flux was determined using isotopomer analysis and O_2 consumption.

2.4. Direct observation of triglyceride turnover

Hearts were cannulated, supplied with non-recirculating KH medium with unenriched fatty acids and all other substrates, and placed in an 8 mm NMR tube. The water-jacketed, two-chamber glass perfusion apparatus was maintained at 37 °C and the entire apparatus fit into the bore of a 14.1 T magnet. During a stabilization time of approximately 20 min, the NMR probe was tuned and the field homogeneity was optimized. After the collection of kinetic data was started, the perfusing medium was switched to a second chamber containing the same substrates but with [U-^{13}C]long-chain fatty acids substituted for unenriched long-chain fatty acids. One heart was freeze-clamped every 8–9 min from 30 to 110 min after switchover, and stored (n = 10 hearts). Frozen tissue was ground into a fine powder under liquid nitrogen, and then the lipids were extracted [27]. Triglyceride content was determined using a colorimetric assay (TR0100, Sigma) [28]. Triglycerides were separated from other lipids on a prepacked silica Sep-Pak column using combinations of methyl-t-butylether and hexene as described by Hamilton and Comai [29]. Another group of five hearts was perfused using the same protocol; however, after the hearts were given [U-^{13}C]long-chain fatty acids for 30 min the perfusate was changed again and 1 μM isoproterenol was added. After NMR data collection for 30 min, the hearts were freeze-clamped and extracted as described above.

2.5. NMR spectroscopy

All spectra were collected using a Varian INOVA 14.1 T spectrometer. WET-HMQC (water suppression enhanced through T1 effects - heteronuclear multiple quantum coherence) NMR spectra of isolated mouse hearts [30] were acquired in an 8 mm inverse detection probe equipped with pulsed field gradient coils (Nalorac, Martinez, CA). An acquisition time of 0.3 s was used and 1823 points were recorded over 6000 Hz. Each spectrum was the sum of 48 transients obtained in 67 s. ^{13}C {^{1}H} NMR spectra of tissue extracts were acquired at 25 °C within a 5 mm Varian broad-band probe using a 45° pulse with a 1.3 s acquisition time and 1 s delay for a total pulse repetition time of 2.3 s, and bi-level WALTZ-16 decoupling [26,30]. The relative areas of the ^{13}C NMR multiplets were used to determine the fractional contribution to acetyl-CoA from each ^{13}C-enriched substrate as well as relative anaplerotic flux [y] [26,31]. ^{1}H NMR spectra of triglycerides extracted from heart tissue extracts were acquired at 600 MHz using a 5 mm indirect detection probe. Spectra were collected using a 90° pulse with a 1 s delay. The ^{13}C satellites in the methyl resonance centered at 0.88 ppm were used to determine triglyceride ^{13}C enrichment.

3. Results

3.1. Sources of acetyl-CoA and citric acid cycle flux

Multiplets due to ^{13}C–^{13}C spin–spin coupling were easily resolved in the ^{13}C NMR spectra of heart extracts (Fig. 1). The contribution of exogenous substrates to acetyl-CoA was fatty acids, 18 ± 6%; ketones, 34 ± 7%; lactate, 24 ± 4%; or pyruvate, 7 ± 4%. (All data are reported as mean ± 1 S.D.) By difference, the contribution of unlabeled acetyl-CoA (from glucose, glycerol, glycogen, and endogenous triglycerides) was 17%. The average anaplerotic flux as a fraction of citric acid cycle flux, y, was 8 ± 3%. In the same group of hearts, O_2 consumption was 18.7 ± 4.1 μmol O_2/min/g dry wt.

The proportionality constant between citric acid cycle flux and oxygen consumption is substrate-dependent due to the differing number of reducing equivalents produced outside the cycle. Since the ^{13}C NMR isotopomer analysis provides the fraction of acetyl-CoA derived from a particular substrate, reducing equivalents produced outside the cycle relative to those produced from the citric acid cycle itself is easily determined [31]. From this information plus the measured oxygen consumption, flux through citrate synthase was 7.2 ± 1.9 μmol/g dry wt/min, and the rate of oxidation of exogenous long-chain fatty acids equivalent to palmitate was (citrate synthase flux) × (fraction of acetyl-CoA derived from fatty acids)/8, or 0.16 μmol/g dry wt/min.

3.2. ^{13}C enrichment in triglycerides by tissue extracts

The concentration of triglycerides in the hearts at the end of the 100-min perfusion period measured chemically was 38 ± 10 μmol/g dry wt. Hearts were freeze-clamped at ~9 min intervals from 30 to 110 min after switching to ^{13}C-enriched fatty acids (n = 10 hearts). The rate of ^{13}C enrichment of the triglyceride pool, measured by ^{1}H NMR of tissue extracts, was approximately linear, % enrichment = $0.18t + 2.9$, $r^2 = 0.43$, where t is time in min from switchover. At 100 min, about 21% of cardiac triglycerides were ^{13}C enriched. Therefore, the rate of appearance of ^{13}C-enriched fatty acids in the triglyceride pool was 0.24 μmol fatty acids/g dry wt/min (equivalent to 0.08 μmol triglycerides/g dry wt/min).

3.3. ^{1}H NMR observation of triglyceride turnover in the beating heart

No signal from fatty acids could be detected from [U-^{13}C]long-chain fatty acids in the medium by WET-HMQC, presumably because of low concentration or NMR "invisibility" due to binding to albumin. Small resonances due to natural abundance ^{13}C in triglycerides were detected in isolated hearts supplied with unlabeled long-chain fatty acids. Hearts were switched to a perfusate containing [U-^{13}C]long-chain

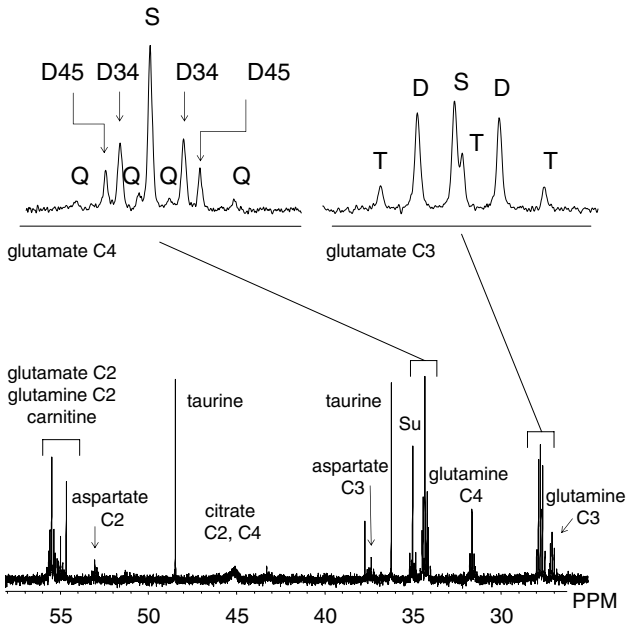

Fig. 1. ^{1}H decouple-^{13}C NMR spectrum of a heart extract. Resonance assignments are shown in the lower panel. Abbreviations: D, doublet in carbon 3 resonance due to either $J_{2,3}$ or $J_{3,4}$; D34, doublet in carbon 4 due to $J_{3,4}$; D45, doublet in carbon 4 due to $J_{4,5}$; S, singlet; Su, succinate; T, apparent triplet in carbon 3 due to $J_{2,3,4}$; Q, quartet or doublet of doublets due to $J_{3,4,5}$.

fatty acids, which over time introduced ^{13}C into the endogenous triglyceride pool. The 1 D WET-HMQC spectrum of labeled triglycerides including chemical shift assignments [32] is shown in Fig. 2 and the full stacked plot is shown in Fig. 3.

The appearance of ^{13}C-enriched fatty acids in the triglyceride pool could represent de novo synthesis or turnover of a triglyceride pool with fixed mass. In separate experiments with hearts supplied with unenriched fatty acids, the natural abundance ^{13}C triglyceride signal was constant over 2 h. In additional studies with hearts supplied with enriched fatty acids, followed by switchover to unenriched fatty acids, the ^{13}C signal remained constant or decreased slightly (data not shown).

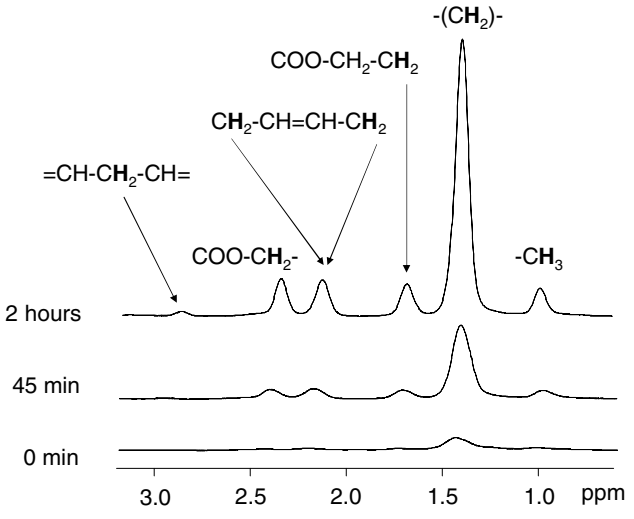

Fig. 2. Selected 1D WET-HMQC spectra of triglyceride region. Each spectrum was acquired over 67 s.

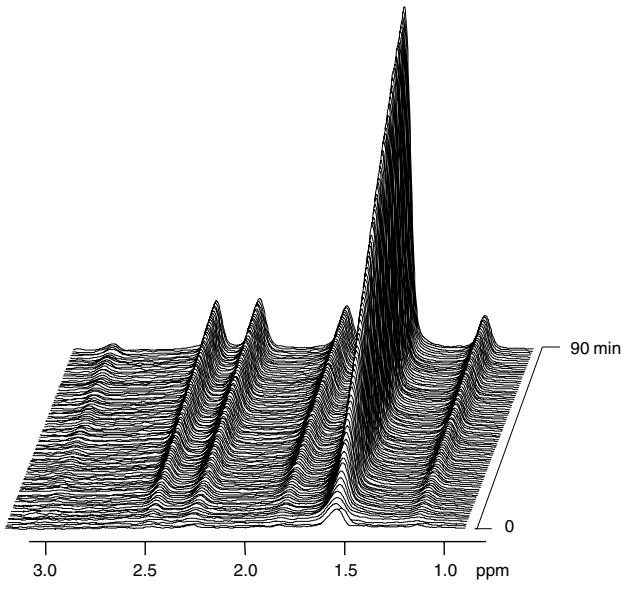

Fig. 3. 1D WET-HMQC over the 2.5–0.9 ppm region from an isolated mouse heart supplied with [U-^{13}C]fatty acids plus unlabeled lactate, pyruvate, glycerol, glucose, and ketones. Chemical shift assignments are shown in Fig. 2.

Therefore, the appearance of ^{13}C-enriched triglycerides reflects turnover in a triglyceride pool of constant mass with rate constant of 0.0024 min^{-1}.

In other studies, addition of isoproterenol (1 μM) caused an increase in heart rate (from 340 to 480 beats/min) and a decrease in the rate of ^{13}C appearance in the triglyceride pool, Fig. 4, to about 33% of the original rate (slope of 1.39 ± 0.07 vs. 0.47 ± 0.15 U/min). In these hearts, fatty acids contributed

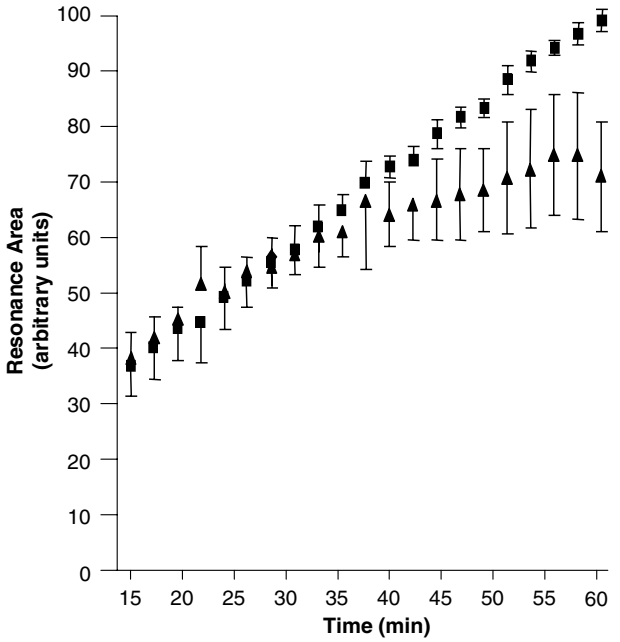

Fig. 4. Enrichment of the triglyceride peaks over the 2.5–0.9 ppm region as monitored by 1D WET-HMQC. Spectra were averaged over 67 s intervals. The first 30 min followed the enrichment in a [U-^{13}C]long-chain fatty acid-perfused heart. The spectra from 30–60 min were obtained after the addition of isoproterenol (triangle) compared to control hearts (square).

27 ± 1% of the total acetyl-CoA, compared to 18% in control hearts.

4. Discussion

These studies demonstrate that triglyceride turnover is easily monitored in the mouse heart using an inverse detection method. Given the small mass of the mouse heart, the observed high signal-to-noise, and the fact that only a small fraction of fatty acids need be enriched to detect triglyceride turnover, this method shows promise for in vivo systems and human applications. Of course there are many potential difficulties of implementing WET-HMQC in vivo, particularly in the moving heart. This technique may be more suitable for relatively homogeneous solid organs such as the liver where abnormal fat stores are critically important in genesis of steatohepatitis and fatty liver disease [33], or in skeletal muscle where abnormal fat metabolism may cause insulin resistance [8]. Conventional ^{13}C NMR isotopomer analysis relies on analysis of the spin–spin coupling patterns that encode metabolic flux information. Unlike this technique, detection of triglyceride kinetics would not depend on replacing a significant fraction of the unlabeled with labeled plasma fatty acids.

In these hearts fatty acids were oxidized at approximately the same rate as incorporation into triglycerides (0.16 and 0.24 μmol/g dry wt/min, respectively). These data indicate that when fatty acids enter the heart and mechanical demand is low, about 60% are esterified and the remaining 40% proceed to direct oxidation in the TCA cycle. The rate of ^{13}C appearance in triglycerides decreased significantly (see Fig. 4) after exposure to isoproterenol. The ^{13}C signal is a function of triglyceride mass, ^{13}C enrichment in the triglycerides, and NMR relaxation times (T_1 and T_2) of the triglycerides. Since serial biochemical measurements of triglyceride mass over time were not practical, it is not possible to distinguish reduction of the mass of stored triglyceride from reduced turnover in the triglyceride pool. Since the fraction of acetyl-CoA derived from fatty acids increased from 18% to 27% after isoproterenol, it is likely that under increased workload, exogenous fatty acids are shunted away from triglycerides and preferentially oxidized.

Direct measurement of acetyl-CoA labeling and triglyceride enrichment demonstrates that the pool of cytosolic fatty acyl-CoA supplying β oxidation is not in isotopic equilibrium with the triglyceride pool. By the end of the perfusion period, triglycerides were enriched to about ~21% in [U-^{13}C]fatty acids. If the entire triglyceride pool was in isotopic equilibrium with cytosolic fatty acyl-CoA, then ~21% of the entire pool would have been ^{13}C enriched while 79%, unenriched. However, combined with the observation that 18% of all acetyl-CoA entering the TCA cycle was derived from ^{13}C-enriched fatty acids and if the entire triglyceride pool is in dynamic equilibrium with long-chain acyl-CoA's contributing to β-oxidation, then fatty acids actually contribute 86% of all of acetyl-CoA entering the TCA cycle (18% from ^{13}C-enriched fats and 68% from unenriched fatty acids). This conclusion is obviously not consistent with the observation that lactate, pyruvate, and ketones contributed 65% of acetyl-CoA. Therefore, one must conclude that the triglyceride pool is not in complete equilibrium with long-chain acyl-CoA's entering the cell and destined for oxidation even after 100 min of continued perfusion. This observation is consistent with numerous previous reports of multiple types of triglyceride pools in heart tissue each characterized by different turnover rates [3,13,34].

Fatty acids generally are considered the preferred substrate for the heart [1,35]. However, some reports concluded that lactate is preferred, based upon suppression of fatty acid utilization after an increase in lactate concentration [36,37], and others [35] find that ketones are the preferred substrate even at the low concentration found in vivo, 0.17 mM. More recently, Ziegler et al. found that ketones at high concentration, 1.6 mM, contributed about 40% of the acetyl-CoA oxidized in the myocardium in vivo [38]. In fact, it is difficult to resolve these competing claims due to variations in substrate concentrations under study and because many early experiments examined competition between only two substrates, presumably because of the technical difficulties in measuring utilization of multiple substrates at once.

This study for the first time employed this group of substrates (acetoacetate, glucose, glycerol, lactate, pyruvate, and long-chain fatty acids) to assess substrate selection in the isolated mouse heart. In contrast to studies that rely on measuring substrate concentration gradients across the heart, tracer dilution, respiratory quotient or $^{14}CO_2$ release, ^{13}C NMR isotopomer analysis unequivocally identifies relative substrate oxidation from multiple substrates simultaneously since it detects the labeling of a metabolic product in the TCA cycle. For example, it is easily seen (Fig. 1) that the contribution of lactate to acetyl-CoA is about twice that of long-chain fatty acids, a result likely due to the concentration of substrates in the perfusate. An additional finding is that acetoacetate is used to a substantial degree, contributing 34% of acetyl-CoA at normal plasma concentrations. The fact that acetoacetate contributed such a high proportion of the oxidized substrate, even at 0.28 mM compared to the relatively high concentration of competing substrates, indicates that ketones are the preferred substrate for energy production in the normal mouse heart in vivo. Studies in rats where with ^{13}C-enriched acetate and ketones were co-infused also found a substantial contribution of ketones to cardiac energy production in vivo [38].

The phrase "substrate preference", widely used in cardiac physiology, is somewhat ambiguous since some studies compared oxidation of two substrates, others examined oxidation of a single substrate in vivo, and others assessed the suppression of competing substrates after the concentration of substrate is increased. Earlier we suggested that a better index may be to compare substrate oxidation on the basis of substrate concentration [35]. In the current study, the ratio (% oxidation)/(concentration) was long-chain fatty acids (29), acetoacetate (121), lactate (4.9), and pyruvate (1.4). After correction for concentration, acetoacetate was overwhelmingly the preferred substrate.

Although earlier reports indicate that isoproterenol results in long-term accumulation of myocardial triglycerides, the present kinetic results shows that isoproterenol slows entry of [U-^{13}C]acyl-CoA into the triglyceride pool of mouse hearts and shifts the fate of [U-^{13}C]acyl-CoA toward β-oxidation likely due to an increased energy demand. These results are not necessarily incongruent because our kinetic data were collected only over a period of ~30 min after isoproterenol administration. Thus, the acute effect of isoproterenol administration appears to be a shift in fatty acid utilization away from triglyceride storage and toward oxidation while the chronic effect likely shifts back toward triglyceride accumula-

tion as the heart adjusts to an increased metabolic energy demand by using other substrates for energy production or because of changes in fatty acid delivery to the heart due to systemic effects of adrenergic stimulation. This apparent dichotomy highlights the importance of dynamic measures of triglyceride turnover using techniques such as that described here.

In summary, inverse detection NMR methods are useful for monitoring the rapid turnover of myocardial triglycerides with high sensitivity and superb temporal resolution. Given the strong interest in understanding the role of triglyceride stores in metabolism and insulin resistance, exploration of these methods in vivo is warranted. In the presence of a mixture of physiological substrates, long-chain fatty acids unexpectedly contributed less to energy production in the mouse heart than is generally accepted.

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